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In Vitro Comparative Study of Osteogenic Differentiation Ability between Adipose and Bone Marrow Mesenchymal Stem Cell Applied to Bovine Demineralized Bone Matrix

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Keywords: bone defect, demineralized bone matrix, mesenchymal stem cell

Abstract. Ideal bone graft must possess the desirable trait such as osteoconductive, osteoinductive and osteogenesis. Demineralized Bone Matrix (DBM) provides both osteoconductive and osteoinductive trait. Referring to the tissue engineering principle, the addition of mesenchymal stem cell would add the osteogenic trait to this procedure. The design of this study is experimental using Bovine DBM. Bone Marrow Mesenchymal Stem Cell (BMSCs) and Adipose Mesenchymal Stem Cells (ASCs) were taken from New Zealand white rabbit. There are two groups of treatment, divided into DBM implanted with BMSCs and DBM implanted with ASCs. Each BMSCs and ASCs groups is incubated in the normal and osteogenic culture plate. Evaluation is performed by counting the osteoblast and immunohistochemistry stain using Alkaline Phosphate and Osteocalcin. After 4 weeks of incubation, we found that the osteoblast count in BMSCs groups is higher compared to the ASCs groups in both culture condition ($p<0.01$) along with Alkaline Phosphate staining ($p<0.05$), while the Osteocalcin staining showed insignificant differences ($p>0.05$). This study revealed that xenogenic bovine DBM can act as the potential osteoinductive scaffold for the MSCs to differentiate. The tissue engineering application by combining MSCs and Bovine DBM can be considered as an alternative in managing bone defect cases.

1. Introduction

Bone defects are one of the major problems in orthopedic cases. It can occur in both trauma and non trauma cases. In bone defects, discontinuities of bone integrity can be caused by high-energy trauma, infection, tumor resection surgery, revision surgery, developmental deformities, and congenital malformation disorders. In this case, the size of the bone defect is too large and is included in the critically sized defect so it will not undergo spontaneous regeneration and require an interventional operation [1,2]. There are 500,000 surgeries that require bone grafting in the United States within a year with a total cost of 300 million USD in 1999 [3]. For comparison, the use of bone graft for orthopedic surgery at Dr. Soetomo General Hospital Surabaya has quadrupled from 1997 to 2001 [4]. Bone defect treatment requires the bone graft to fill the gap and autologous bone graft is still a gold standard in the treatment of bone defect cases [5]. However, postoperative pain in bone donor source areas is of concern in the use of autologous bone graft besides its limitations of the bone sources [6]. Other alternatives for the graft subtitutes are allogenic bone graft and animals xenogenic bone graft. Allograft is physiologically more resembling the original bone and may have osteoinductive properties but the donor availability is also limited and disease transmission still be concerned. Whereas xenograft, owing to the abundance of donors, may be less expensive and more readily available even though its properties are limited to osteoconductive alone [7].

With the rapid progress in the concept of tissue engineering, the use of stem cells that originally are exogenous cells in bone tissue engineering serves osteogenic capacity in stimulating bone healing [8,9]. Together with the development of scaffolding techniques by performing demineralization so cellular components that potentially induce immune response can be eliminated, thus providing potential scaffold as a microenvironment of the stem cell [8].

Considering this, bovine Demineralized Bone Matrix (DBM) which is a xenogenic bone graft product can be rated as the alternative bone graft substitues, because its osteoconductive and osteoinductive properties can serve as a scaffold to support stem cell proliferation and differentiation thus provide osteogenic capacity [9]. Besides its economically cost, bovine DBM has been shown to have higher bone healing promotion capacities than the other type of bone graft and has lower immunogenicity and rejection reactions to surrounding tissue [10]. This current study will test and compare the ability of bovine DBM as scaffold and growth factor provider to stimulate differentiation of bone marrow and adipose mesenchymal stem cells to enhance its function as a bone graft.

2. Materials and Methods

This study was an in vitro laboratory study conducted at the Institute of Tropical Disease (ITD) Airlangga University and Tissue Bank of Dr. Soetomo General Hospital in Surabaya, Indonesia. The experimental unit was BMSCs and ASCs isolated from a healthy male New Zealand white rabbit 8 months old and weighing 3.0 kg approved by Airlangga University and Dr. Soetomo General Hospital research committee. In this study, bovine DBM added to isolated and replicated BMSCs and ASCs and divided into 4 groups (Figure 1).

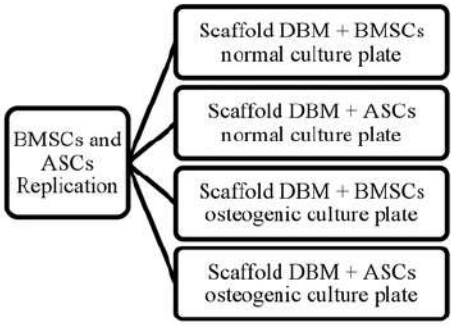

Figure 1. Research design

2.1 Preparation of bovine demineralized bone matrix

Demineralized bone matrix, prepared from the femoral condyle of the 2-year-old local cow, were collected from the local slaughterhouse. All bones were collected aseptically, and the soft tissues were removed before storage at -70°C. The bones were later cleared of fascia and cut into 1-cm pieces with a bone saw under saline (0.9% NaCl) solution lavage. Bone pieces were stored at -70°C until further use. The pieces were then thawed in 95% ethanol for 15 minutes and air dried. All bones were milled to 10 mm pieces. The pieces were then decalcified in 0.6 mol/l HCl at 4°C for 8 days under constant agitation. After demineralization, all bone pieces were rinsed in sterile water and placed in phosphate buffer overnight. The bone pieces were then rinsed and the pH was adjusted to 7.3. They were placed in ethanol, the ethanol was allowed to evaporate overnight, and the pieces were packaged aseptically and stored at 4°C.

2.2 Isolation of bone marrow mesenchymal stem cells

Animals were anesthetized with ketamine (40 mg/kg, IM) and xylazine (5 mg/kg, IM). Isolation of MSCs from bone marrow performed aseptically by cleaning the skin of rabbit femur. The muscle and connective tissue were removed from the femur by scraping the diaphysis of the bone clean

then pulling the tissue toward the ends of the bone. A 27-gauge needle was inserted and flushed with Dulbecco's Modified Eagle's Medium (DMEM) and collect in a 15mL tube. The cell suspension was filtered through a 70-μm filter mesh. Bone marrow cells were cultured in DMEM+10% FBS+1% antibiotic-antimycotic solution tissue culture flask and incubated at 37°C with 5% CO_2. For osteogenic culture, 10 mmol dexamethasone, 10 mmol β-glycerol-phosphate, 0.05 mmol 2-phosphate ascorbic acid, 100 U/mL penicillin/streptomycin, and 10 mmol 1,25-dihydroxy vitamin D3 were added to culture plate.

2.3 Isolation of adipose mesenchymal stem cells

Isolation of MSCs from adipose tissue was performed by subcutaneous fat pad liposuction under sterile conditions and washed with phosphate-buffered saline (PBS) containing 1% antibiotic-antimycotic solution. The washing step was repeated until all blood vessels and connective tissues appeared to have been liberated. Adipose tissue then minced into small pieces and digested in 0.1% type 1 collagenase at 37°C with shaking for 2 hours and added 5mL DMEM containing 10% fetal bovine serum. For further disintegration of tissue aggregates, the sample was pipetted up and down several times. The cell suspension was filtered through 100 μm filter for the removal of the solid aggregates. The sample was subsequently centrifuged at 2000 rpm for 5 minutes at room temperature to complete the separation of the stromal cells from the adipocytes and the supernatant removed without disturbing the cells. The pellet was resuspended in 1 ml of lysis buffer to lyse red blood cells, incubated for 10 minutes, washed with 10 ml of PBS+1% antibiotic-antimycotic mixture and centrifuged at 2000 rpm for 5 minutes. The supernatant was removed and the cell pellet was resuspended in complete medium (DMEM with 20% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution) in a 25 cm^2 culture flask and maintained in an incubator supplied with the humidified atmosphere of 5% CO_2 at 37°C.

2.4 Cell cultivation

After one day, non-adherent cells were removed by two to three washes with PBS and adherent cells further cultured in complete medium. The medium was changed every 3 days until the monolayer of adherent cells reached 70-80% confluence. Cell passaging was performed using trypsin-EDTA solution 0.25%. Cell cultivation was performed up to the 3rd passage.

2.5 Osteogenic differentiation

Passage 3 MSCs were harvested by trypsin digestion as described above. The cells were counted and seeded at a density of 5×10^4 per well in a 9-well plate each group. The medium was changed twice per week for 2-3 weeks. The differentiation potential for osteogenesis was observed by the number of osteoblasts, alkaline phosphatase (ALP), and osteocalcin (OC). The number of osteoblasts assessed by hematoxylin-eosin staining, osteogenic marker gene of alkaline phosphatase and osteocalcin assessed by immunohistochemical staining under immunofluorescence microscopy. The Immunoreactive Score (IRS) by Remmele is a scale index is used to quantify the immunohistochemistry findings resulted by multiplication of immunoreactive cell percentage scores with color intensity scores on immunoreactive cells (Table 1). The data of each microscopic examinations were observed in 5 fields of view.

Table 1. The semi-quantitative scale of the IRS (IRS result = AxB)

A	B
Score 0: no positive cells	Score 0: no color reactions
Score 1: positive cells less than 10%	Score 1: low color intensity
Score 2: positive cells ranging from 11% - 50%	Score 2: medium color intensity
Score 3: positive cells between from 51% - 80%	Score 3: strong color intensity
Score 4: positive cells between from more than 80%	

3. Results and Discussion

The MSCs cultures were observed by using an inverted light microscope. Adherence of spindle-shaped cells to culture plastic flask was observed after 1 day of culture for both BMSCs and ASCs. Primary cultures reached 70-80% confluence faster in BMSCs than for ASCs (Figure 2).

Figure 2. (A) Bone marrow stem cells culture, **(B)** Adipose stem cells culture

Table 2 and **Figure 3** reveal the mean total number of osteoblast cells in the BMSCs group added with bovine DBM and cultured at normal condition was 7.67±2.00 cells per 5 fields of view, higher than the osteoblast cell number in the ASCs group (1.44±1.24) and the difference is statistically significant (p = 0.000). Similiar significant result found for the mean of osteoblast number cells in the osteogenic culture, BMSCs group was 120.67±28.54 compared to ASCs group 73.00±42.32 cells number per 5 fields of view (p = 0.013).

Table 2. Osteogenic differentiation of mesenchymal stem cells

	BMSCs (Mean ± SD)		ASCs (Mean ± SD)	
	Culture Condition			
	Normal	Osteogenic	Normal	Osteogenic
Osteoblast Count (Cell number/ 5 FV)	7.67±2.00	120.67±28.54	1.44±1.24	73.00±42.32
ALP Expression (IRS Score)	3.20±0.63	3.84±0.53	1.12±0.36	1.16±0.33
Osteocalcin Expression (IRS Score)	1.96±0.59	3.16±0.45	2.18±0.75	3.29±0.61

The average immunoreactive score (IRS) of ALP expression in BMSCs group added with bovine DBM within normal culture was 3.20±0.63 compared to 1.12±0.36 in ASCs groups and had a statistically significant effect (p = 0.000). Thus in osteogenic culture, the IRS mean of the ALP expression in the BMSCs group compared to ASCs group added with bovine DBM also differ significantly (p = 0.000).

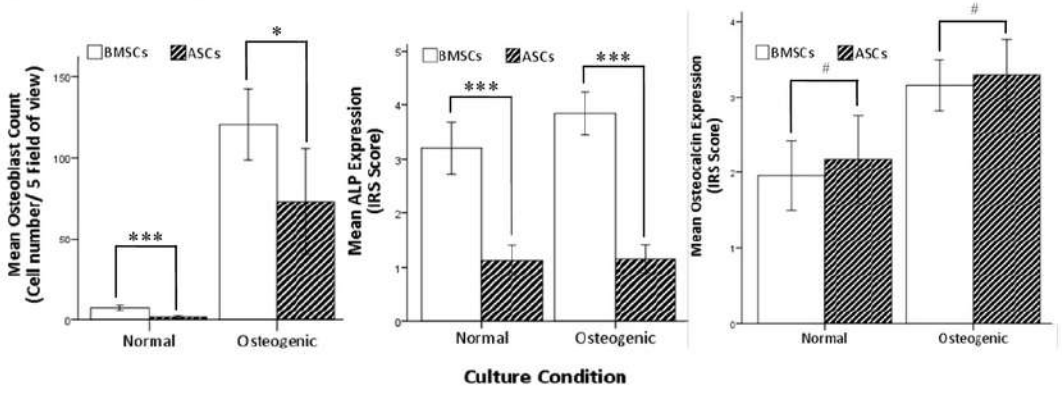

Figure 3. Number of osteoblast cell, immunoreactive score of ALP and osteocalcin for BMSCs and ASCs within normal and osteogenic culture, (*=p<0.05 **=p<0.01 *** p<0.001 #=p>0.05)

The mean immunoreactive score of OC expression in the BMSCs group added with bovine DBM and cultured at normal condition was slightly lower than in the ASCs group (1.96±0.59 *vs* 2.18±0.75) but not statistically significant (p = 0.498). Whereas the average IRS of osteocalcin expression at osteogenic culture in BMSCs group was 3.16±0.45 and 3.29±0.61 in ASCs group but the results were not statistically significant (p = 0.603).

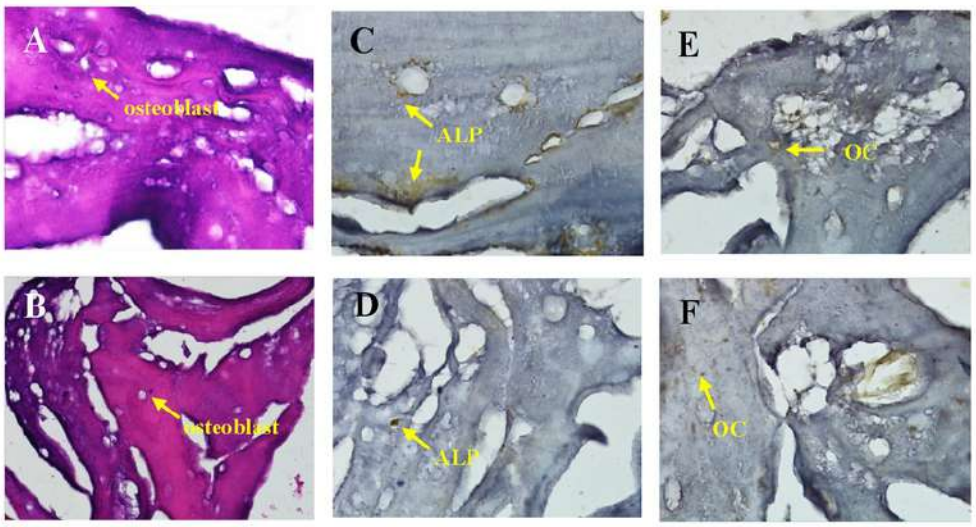

Figure 4. **(A)** HE staining on normal culture of BMSCs, **(B)** HE staining on normal culture of ASCs, **(C)** ALP expression in normal culture of BMSCs, **(D)** ALP expression in normal culture ASCs tissue, **(E)** OC expression in normal culture of BMSCs tissue, **(F)** OC expression result in normal culture ASCs tissue.

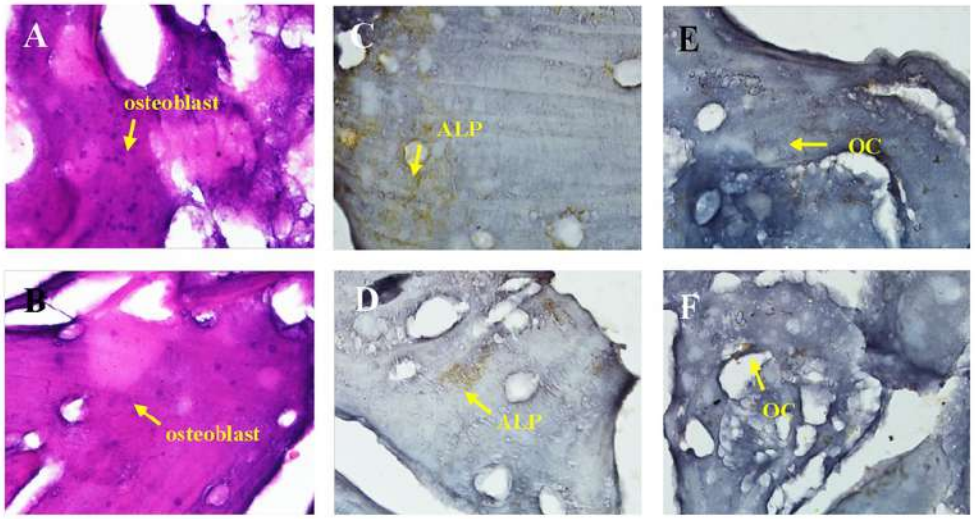

Figure 5. **(A)** HE staining on osteogenic culture of BMSCs. **(B)** HE Staining on osteogenic culture of ASCs **(C)** ALP expression in osteogenic culture of BMSCs, **(D)** ALP expression in osteogenic culture ASCs tissue, **(E)** OC expression in osteogenic culture of BMSCs, **(F)** OC expression result in osteogenic culture ASCs.

The use of stem cells in combination with scaffolds showed mixed results. The capability of mesenchymal stem cell differentiation (MSC) largely determines its use in managing various tissue damage. Stem cells derived from bone marrow are MSCs which was first studied and used widely. However, the morbidity induced when taking BMSCs causes other sources such as adipose and umbilical cord mesenchymal stem cells is further investigated [11]. Although most researchers conclude that BMSCs have better osteogenic proliferation capabilities than others [12-14], Kern et

al investigated the differentiation of bone marrow, adipose, and umbilical cord mesenchymal stem cells, became bone, and the results showed no significant difference between BMSCs and ASCs [15]. This led to the study using both types of stem cells growing at a rapid rate to handle orthopedic cases with bone defects.

Studies by Garmie et al and Janicki et al showed promising results from stem cell and scaffold applications of non-connective fractures [16,17]. However, this study supported by several other studies, shows that BMSCs remain superior to muscle, adipose and umbilical cord [18-20]. Other studies have suggested the use of embryonic stem cells with scaffolds with promising results, but limited cell resources and difficulty in processing require further study to obtain optimal results [21]. Liu et al used ASCs inserted into the scaffold of the shells to correct the cranial defects in experimental animals with satisfactory results [22].

Several experiments have been conducted to combine DBM with MSC. Schubert et al reported a significant difference between the use of ASCs implanted into DBM compared with the autopsy of the cancellous bones in spinal fusion using pig experiments [23]. Evaluations performed using CT scans and histologic examinations showed better performance in the group DBM added A-MSCs. Several other researchers have tried to combine DBM with BMSCs with satisfactory results [24,25].

The results of this study indicate a significant comparison in which the amount of osteoblasts and alkaline phosphatase expression produced by differentiation of BMSCs is higher than ASCs, but the resulting osteocalcin expression did not differ significantly. This is in line with the previous hypothesis that BMSCs have a higher osteogenic differentiation potential than ASCs. This study also proves that bovine DBM is able to function as a scaffold where stem cells differentiate into osteoblasts.

Studies using ASCs and the scaffold, both DBM and other scaffold forms, are still widely used to determine their efficacy in overcoming bone defects, as the use of BMSCs means increasing morbidity in patients in the form of pain in bone marrow collection sites. The authors argue that in vivo research is needed to directly compare the efficacy of DBM use in the treatment of bone defects in addition to ASCs or BMSCs.

4. Conclusion

Xenogenic bovine Demineralized Bone Matrix (DBM) is able to function as a scaffold where mesenchymal stem cells differentiate into osteoblast cells. Bone marrow mesenchymal stem cells have better osteogenic differentiation capabilities than adipose mesenchymal stem cells when implanted into Demineralized Bone Matrix.

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